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METHODS FOR ACUTE TOXICITY TESTS WITH FISH, MACROINVERTEBRATES, AND AMPHIBIANS

NATIONAL WATER QUALITY LABORATORY

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METHODS FOR ACUTE TOXICITY TESTS WITH FISH, MACROINVERTEBRATES, AND AMPHIBIANS

by

The Committee on Methods for Toxicity Tests
with Aquatic Organisms

Program Element 1BA021

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Four detailed methods for conducting acute toxicity tests with freshwater, estuarine, and marine fish, macroinvertebrates, and amphibians are presented in an integrated format. Nomenclature is consistent with that used in other branches of toxicology. Concepts incorporated into the methods are applicable to toxicity tests with most aquatic organisms.

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SECTION I

CONCLUSIONS

1. A diverse group of scientists interested in aquatic toxicology can reach agreement concerning detailed methods for conducting toxicity tests with aquatic animals.
2. Many of the methods written in the past for conducting toxicity tests with aquatic organisms were not as detailed or as consistent as desirable.
3. Most acute toxicity tests can be conducted according to uniform, detailed methods.

SECTION II

RECOMMENDATIONS

1. Toxicity tests with aquatic organisms should be conducted according to uniform, detailed methods whenever possible to maximize the number of reliable comparisons that can be made concerning relative toxicity and relative sensitivity.
2. Whenever toxicity tests are conducted with aquatic organisms, the methods presented herein should be followed as closely as possible.
3. Reports of toxicity tests should contain all information necessary to allow correct use of the results.
4. More effort should be expended toward writing a consistent set of detailed methods for toxicity tests with aquatic organisms by a procedure that allows input by all interested persons from academic, industrial, contract, regulatory, and other organizations.
5. Additional research on methodology is needed to identify improvements that should be made in the present methods. In particular, research should be directed toward identifying means of determining the overall quality or "healthiness" of aquatic animals used for toxicity tests.
6. To obtain the most meaningful data from a toxicity test with aquatic organisms, the investigator should consult with an aquatic biologist, analytical chemist, biometrician, and aquatic toxicologist before the test.
7. Aquatic toxicologists should regularly exchange information concerning methodology with each other and with other toxicologists in the United States and in other countries and should cooperate to avoid proliferation of committees writing methods.

SECTION III

INTRODUCTION

A. BACKGROUND

The Committee on Methods for Toxicity Tests with Aquatic Organisms was organized in November, 1971, at the suggestion of Mr. Andrew J. Culver, Jr., to foster cooperation, uniformity, and excellence in the field of aquatic toxicology. The members of the committee and the organizations with which they were affiliated are:

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To help the committee in its work, a Subcommittee on Effluent Tests was organized with the following members:

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In addition, many other people were contacted in the organizations of the members of the committee and subcommittee and through a mailing list of about 150 names and all known existing methods were reviewed. Although the methods presented herein were written mainly by and for aquatic toxicologists in the United States, to encourage international cooperation comments were solicited and received from scientists in other countries, particularly Canada and Great Britain. Although the committee gratefully acknowledges helpful advice from many people, it accepts full responsibility for the methods contained herein.

The committee felt that an immediate need existed for detailed methods for conducting toxicity tests with aquatic organisms. Therefore it undertook the task of writing some such methods in order to make recommendations for conducting such tests to give guidance to those who desire it, to discourage the use of methods considered unacceptable, and to encourage uniformity in methodology and nomenclature so that the results of toxicity tests with aquatic organisms would be more useful. Nomenclature consistent with that used in other branches of toxicology was used whenever possible to facilitate communication between aquatic and other toxicologists.

Because of the formation of sections dealing with aquatic toxicology within Committee E-35 on Pesticides and Committee D-19 on Water of the American Society for Testing and Materials (ASTM), the Committee on Methods for Toxicity Tests with Aquatic Organisms is dissolving with the publication of this document to help prevent a proliferation of competing committees and conflicting methods.

B. NOMENCLATURE

In a toxicity test two or more treatments are used to study the effect of a toxic agent on test organisms which are usually all of the same

species. Although toxicity tests with aquatic organisms can be conducted by applying the toxic agent directly to the test organisms, such as by injection or in food, most tests are conducted by exposing the test organisms to test solutions containing various levels of a toxic agent. One or more control treatments are used to provide a measure of the acceptability of the test by giving some indication of the healthiness of the test organisms and the suitability of the dilution water, test conditions, handling procedures, etc. A control treatment is an exposure of the test organisms to dilution water with no toxic agent added. The other treatments are exposures of the test organisms to dilution water with toxic agent added. The toxic agent can be one or more pure chemicals or a complex mixture such as a formulation or an effluent. Sometimes the test solutions are not true solutions because they contain undissolved toxic agent. Test solutions are often prepared by dissolving a toxicant in a solvent, preferably water, to form a stable stock solution, and then adding a portion of the stock solution to dilution water. Generally the most important data obtained from a toxicity test are the percentages of test organisms that are affected in a specified way by each of the treatments. The result derived from these data is a measure of the toxicity of the toxic agent to the test organisms under the conditions of the test or, in other words, a measure of the susceptibility of the test organisms to the toxic agent.

Acute toxicity tests are generally used to determine the level of toxic agent that produces an adverse effect on a specified percentage of the test organisms in a short period of time. Because death is normally an easily detected and obviously important adverse effect, the most common acute toxicity test is the acute mortality test. Experimentally, 50% effect is the most reproducible measure of the toxicity of a toxic agent to a group of test organisms, and 96 hours is often a convenient, reasonably useful exposure duration. Therefore, the measure of acute toxicity most often used with fish, macroinvertebrates, and amphibians

is the 96-hour median lethal concentration (96-hr LC50). Thus the result of an acute mortality test is the statistically derived best estimate of the LC50, which is the concentration of toxicant in dilution water that is lethal to exactly 50% of the test organisms during continuous exposure for a specified period of time, based on data from one experiment. However, the measure of acute toxicity most often used with daphnids and midge larvae is the 48-hour median effective concentration (48-hr EC50) based on immobilization. The terms median lethal concentration (LC50) and median effective concentration (EC50) are consistent with the widely used terms median lethal dose (LD50) and median effective dose (ED50), respectively. However, whereas "concentration" refers to the concentration of toxicant in the test solution, "dose" refers to the amount of toxicant that enters the test organism. For toxic agents or tests to which neither concentration nor dose applies, such as tests with temperature, the terms median lethal level (LL50) and median effective level (EL50) should be used.

Acute toxicity tests in which test organisms are exposed to test solutions containing a toxic agent can be conducted by at least four techniques:

1. In the static technique test solutions and test organisms are placed in test chambers and kept there for the duration of the test.
2. The recirculation technique is like the static technique except that each test solution is continuously circulated through an apparatus to maintain water quality by such means as filtration, aeration, and sterilization and then returned to the test chamber.
3. The renewal technique is like the static technique except that the test organisms are periodically exposed to fresh test solution of the same composition, usually once every 24 hours, either by transferring the test organisms from one test chamber to another or by replacing the test solution.

4. In the flow-through technique test solutions flow into and out of the test chambers on a once through basis for the duration of the test. Two procedures can be used. In the first large volumes of the test solutions are prepared before the beginning of the test and these flow through the test chambers. In the second and more common procedure fresh test solutions are prepared continuously or every few minutes in a toxicant delivery system.

With any of these techniques a pump or stirrer can be used to create a current in the test chambers to accommodate particular test organisms, but this will often increase aeration and volatilization.

SECTION IV

METHODS

A. PREFACE

The BASIC STATIC ACUTE TOXICITY TEST METHOD, BASIC FLOW-THROUGH ACUTE TOXICITY TEST METHOD, EFFLUENT STATIC ACUTE TOXICITY TEST METHOD, and EFFLUENT FLOW-THROUGH ACUTE TOXICITY TEST METHOD are presented herein in a format that eliminates repetition and does not require referencing from one method to another. Items that do not apply to all four methods are clearly labelled according to the methods to which they do apply. The BASIC test methods are the ones that aquatic toxicologists will normally use when conducting acute toxicity tests. When special situations arise, the BASIC test methods can be modified to meet special needs. The most important special need at this time is effluent testing and so two EFFLUENT test methods were written by making appropriate modifications in the BASIC test methods.

B. EQUIPMENT

1. Facilities

The facilities should include tanks for holding and acclimating test organisms, and a constant-temperature area or recirculating water bath for the test chambers. For STATIC tests there should be a dilution-water tank that may be used to prepare reconstituted water and is sometimes elevated so dilution water can flow by gravity into holding and acclimation tanks and test chambers. For FLOW-THROUGH tests there should be an elevated headbox so dilution water can flow by gravity into

holding and acclimation tanks and the toxicant delivery system. For FLOW-THROUGH tests ceilings should be at least 10 feet high to accommodate proportional diluters, and strainers and air traps should be included in the water supply system. Holding, acclimation, and dilution-water tanks and headboxes should be equipped for temperature control and aeration. Air used for aeration must be free of oil and fumes; filters to remove oil and water are desirable. During holding, acclimation, and testing, test organisms should be shielded from disturbances. The test facility must be well ventilated and free of fumes. For BASIC tests, a 16-hour light and 8-hour dark photoperiod should be provided with a 15- to 30-minute transition period controlled by a system such as that described by Drummond and Dawson (1970).

Organisms requiring special conditions must be accommodated during holding, acclimation, and testing. For example, burrowing mayfly nymphs should be provided a burrowing substrate, such as that described by Fremling and Schoening (1973) and Fremling (1974); immature stream insects should always be in a current in a system such as that described by Nebeker and Lemke (1968); and penaeid shrimp and bottom-dwelling fish should be provided a silica sand substrate. Since cannibalism can occur among many species of decapods, the claws of crabs and crayfish should be banded or the individuals should be physically isolated, by such means as screened compartments.

2. Construction Materials

Construction materials and commercially purchased equipment that may contact any water into which test organisms are placed should not contain any substances that can be leached or dissolved by the water. In addition, materials and equipment that contact stock solutions or test solutions should be chosen to minimize sorption of toxicants from water. To minimize leaching, dissolution, and sorption, glass, #316 stainless

steel, and perfluorocarbon plastics must be used whenever possible. Unplasticized plastics can be used for holding and acclimation tanks and in the water supply system. Rubber, copper, brass, and lead must not come in contact with dilution water, stock solutions, effluent samples, or test solutions.

3. Toxicant Delivery Systems for FLOW-THROUGH Tests

Although many toxicant delivery systems can be used (Lowe, 1964; Sprague, 1969; Freeman, 1971; Cline and Post, 1972; Grammo and Kollberg, 1972; Bengtsson, 1972; Lichatowich et al., 1973; Shumway and Palensky, 1973; Abram, 1973; Schimmel, Hansen, and Forester, 1974; D. DeFoe, National Water Quality Laboratory, Duluth, Minnesota, personal communication; R. Garton, Western Fish Toxicology Station, Corvallis, Oregon, personal communication), the proportional diluter (Mount and Brungs, 1967) is probably the best for routine use. It is accurate over extended periods of time, is nearly trouble-free, and has fail-safe provisions. A small chamber to promote mixing of toxicant-bearing water and dilution water should be used between the diluter and the test chambers for each concentration. If duplicate test chambers are used, separate delivery tubes must be run from this mixing chamber to each duplicate. Alterations in the design of the proportional diluter, such as the use of six or more concentrations, have been useful in some situations (Esvelt and Connors, 1971; McAllister, Mauck, and Mayer, 1972; Benoit and Puglisi, 1973; Chandler, Sanders, and Walsh, 1974; D. Allison, National Water Quality Laboratory, Duluth, Minnesota, personal communication; S. Schimmel et al., Gulf Breeze Environmental Research Laboratory, Gulf Breeze, Florida, personal communication; V. Snarski and F. Puglisi, National Water Quality Laboratory, Duluth, Minnesota, personal communication). The flow rates through the test chambers must be five water volumes per 24 hours for daphnids, and must be at least five water volumes per 24 hours for all other animals. It is usually desirable to construct the

toxicant delivery system so that it can provide at least ten water volumes per 24 hours. The flow rates through the test chambers should not vary by more than 10% from any one test chamber to any other or from one time to another within a test.

For high concentrations of effluent approaching 100%, it might be desirable to use paired flows (Jackson and Brungs, 1967; American Public Health Association, 1971) but pumps are sometimes unreliable and small tubes and openings tend to clog, especially in effluent testing. Alternatively, the proportional diluter (Mount and Brungs, 1967) can be modified by eliminating the W-1 cell and the M-1 cell and introducing 100% effluent directly into the C-2 cell.

The calibration of the toxicant delivery system should be checked carefully before and after each test. This should include determining the flow rate through each test chamber and measuring either the concentration of toxicant in each test chamber or the volume of solution used in each portion of the toxicant delivery system. The general operation of the toxicant delivery system should be checked daily during the test.

4. Test Chambers

Test chambers can be made by welding, not soldering, stainless steel or by gluing double-strength or stronger window glass with clear silicon adhesive. Silicon adhesive sorbs some organochlorine and organophosphorus pesticides which are then difficult to remove. Therefore, as little of the adhesive as possible should be in contact with water; extra beads of adhesive should be on the outside of chambers rather than on the inside. For larger organisms (over 0.5 g each) the test solution should be between 15 and 30 cm deep.

For STATIC tests larger organisms are often exposed in 19.6-liter (5-gallon) wide-mouth soft-glass bottles containing 15 liters of solution (Hesselberg and Burress, 1967) or in 30 cm X 60 cm X 30 cm deep all-glass test chambers. Smaller organisms are often exposed in 3.9-liter (1-gallon) wide-mouth soft-glass bottles or battery jars containing 2 to 3 liters of solution. Daphnids and midge larvae are often exposed in loosely covered 250-ml beakers containing 200 ml of solution.

For FLOW-THROUGH tests test chambers can be made by modifying glass bottles, battery jars, or beakers to provide screened overflow holes or V-notches. Larger organisms are often exposed in 30 liters of solution in a 30 cm X 60 cm X 30 cm deep all-glass test chamber in BASIC tests and in 15 to 30 liters of solution in EFFLUENT tests. Smaller organisms are often exposed in 2 to 4 liters of solution.

5. Cleaning

Toxicant delivery systems and test chambers must be cleaned before use. New ones must be washed with detergent and rinsed with 100% acetone, water, acid (such as 5% concentrated nitric acid), and twice with tap or other clean water. At the end of every BASIC test, if the toxicant delivery system or test chambers are to be used again, they should be (a) emptied, (b) rinsed with water, (c) cleaned by a procedure appropriate for removing the toxicant tested (e.g., acid to remove metals and bases; detergent, organic solvent, or activated carbon to remove organic compounds), and (d) rinsed twice with water. At the end of every EFFLUENT test, if the toxicant delivery system or test chambers are to be used again, they must be cleaned the same as new ones. Acid is useful for removing mineral deposits, and 200 mg of hypochlorite/liter is useful for removing organic matter and for disinfection. However, acid and hypochlorite must not be used together. Test chambers and toxicant delivery systems must be rinsed with dilution water just before use.

C. DILUTION WATER

1. General Requirements

An adequate supply of a dilution water that is acceptable to the test organisms and the purpose of the test must be available. For acute toxicity tests a minimal criterion for an acceptable dilution water is that healthy test organisms will survive in it for the duration of acclimation and testing without showing signs of stress, such as discoloration or unusual behavior. Because daphnids are more sensitive to many toxicants than most other freshwater aquatic animals, a more realistic criterion for an acceptable freshwater dilution water is that first instar daphnids will survive in it for 48 hours without food. A more stringent criterion for an acceptable dilution water is that test organisms will survive, grow, and reproduce satisfactorily in it. Water in which daphnids will survive and reproduce satisfactorily should be an acceptable dilution water for most tests with freshwater animals.
For BASIC tests, the dilution water should be intensively aerated by such means as air stones, surface aerators, and screen tubes (Rucker and Hodgeboom, 1953; Wm. Spoor, National Water Quality Laboratory, Duluth, Minnesota, personal communication) prior to the introduction of the toxicant. Adequate aeration will bring the pH and the concentration of dissolved oxygen and other gases into equilibrium with air, and minimize oxygen demand and the concentration of volatiles. For BASIC tests, the concentration of dissolved oxygen in the dilution water should be between 90% and 100% saturation, and water that may be contaminated with undesirable microorganisms should be passed through a properly maintained ultraviolet sterilizer equipped with an intensity meter.

2. Reconstituted Water

The recommended reconstituted waters (Tables 1 and 2) should be used as the dilution water for as many BASIC tests as possible to maximize the

number of reliable comparisons that can be made concerning relative toxicity and relative sensitivity. Reconstituted water is prepared by adding known amounts of specified reagent-grade chemicals to water which meets the following specifications:

Specific conductance	<1 micromho/cm
Total organic carbon (TOC) or chemical oxygen demand (COD)	<1 mg/l <2 mg/l
Boron, fluoride	<100 µg/l each
Un-ionized ammonia	<20 µg/l
Aluminum, arsenic, chromium, cobalt, copper, iron, lead, nickel, zinc	<1 µg/l each
Residual chlorine	<3 µg/l
Cadmium, mercury, silver	<100 ng/l each
Total organophosphorus pesticides	<50 ng/l
Total organochlorine pesticides plus polychlorinated biphenyls (PCB's)	<50 ng/l

Distilled water and carbon-filtered deionized water are generally acceptable, but the specific conductance must be measured on each batch from which reconstituted water is to be prepared, and the other characteristics must be measured at least twice a year and whenever significant changes in these characteristics are expected. If the water is prepared from a surface water, TOC, or COD must be measured on each batch. If the water is prepared from a chlorinated water, residual chlorine must be measured on each batch or it must be shown that first instar daphnids can survive in each batch of reconstituted water for 48 hours without food.

Whenever possible, the soft reconstituted fresh water (Table 1a) should be used for BASIC tests with freshwater animals. The other reconstituted fresh waters (Tables 1a and 1b) should be used for studying the effects of water quality on the results of toxicity tests. However, the buffers used in Table 1b may react chemically with some toxicants.

Whenever possible, reconstituted sea water (Table 2) of 34 g/kg (ppt, °/oo) salinity and pH 8.0 should be used for BASIC tests with true marine stenohaline species, and 25 g/kg salinity and pH 8.0 with euryhaline species. Other salinities can be used for studying the effects of water quality on the results of toxicity tests. The initial salinity of the reconstituted sea water given in Table 2 is 34 $\pm$ 0.5 g/kg, and the desired test salinity is attained at time of use by dilution with water that meets the specifications listed above.

All reconstituted waters should be intensively aerated prior to use, except that the buffered soft fresh waters listed in Table 1b should be aerated before but not after the addition of the buffer chemicals listed therein.

3. Alternative Dilution Waters

Use of a reconstituted water for FLOW-THROUGH tests is generally impractical. Alternative dilution waters should be uncontaminated and constant quality and should meet the following specifications:

Suspended solids	<20 mg/l
TOC	<10 mg/l
Un-ionized ammonia	<20 µg/l
Residual chlorine	<3 µg/l
Total organophosphorus pesticides	<50 ng/l
Total organochlorine pesticides plus PCB's	<50 ng/l

Table 1. RECOMMENDED RECONSTITUTED FRESH WATERS[a]

Table 1a. Quantities of reagent-grade chemicals required to prepare recommended reconstituted fresh waters and the resulting water qualities.

Name	Salts required (mg/l)				pH[b]	Hardness[c]	Alkalinity[c]
	$NaHCO_3$	$CaSO_4 \cdot 2H_2O$	$MgSO_4$	KCl			
Very soft	12	7.5	7.5	0.5	6.4-6.8	10-13	10-13
Soft	48	30.0	30.0	2.0	7.2-7.6	40-48	30-35
Hard	192	120.0	120.0	8.0	7.6-8.0	160-180	110-120
Very hard	384	240.0	240.0	16.0	8.0-8.4	280-320	225-245

Table 1b. Quantities of reagent-grade chemicals to be added to aerated soft reconstituted fresh water for buffering pH. The solutions should not be aerated after addition of these chemicals.

pH[d]	Milliliters of solution for 15 liters of water		
	1.0 N NaOH	1.0 M KH_2PO_4	0.5 M H_3BO_3
6.0	1.3	80.0	---
6.5	5.0	30.0	---
7.0	19.0	30.0	---
7.5	---	---	---
8.0	19.0	20.0	---
8.5	6.5	---	40.0
9.0	8.8	---	30.0
9.5	11.0	---	20.0
10.0	16.0	---	18.0

[a]From Marking and Dawson (1973).
[b]Approximate equilibrium pH after aeration and with fish in water.
[c]Expressed in mg/l as $CaCO_3$.
[d]Approximate equilibrium pH with fish in water.

Table 2. RECOMMENDED PROCEDURE FOR PREPARING RECONSTITUTED SEA WATER[a]

Add the following reagent-grade chemicals in the amounts and order listed to 890 ml water. Each chemical must be dissolved before another is added.

Chemical	Amount
NaF	3 mg
$SrCl_2 \cdot 6H_2O$	20 mg
H_3BO_3	30 mg
KBr	100 mg
KCl	700 mg
$CaCl_2 \cdot 2H_2O$	1.47 g
Na_2SO_4	4.00 g
$MgCl_2 \cdot 6H_2O$	10.78 g
NaCl	23.50 g
$Na_2SiO_3 \cdot 9H_2O$	20 mg
Na_4EDTA[b]	1 mg
$NaHCO_3$	200 mg

If the resulting solution is diluted to 1 liter, the salinity should be 34 ± 0.5 g/kg and the pH 8.0 ± 0.2. The desired test salinity is attained by dilution at time of use.

[a]From Kester et al. (1967), Zaroogian et al. (1969), and Zillioux et al. (1973).

[b]Tetrasodium ethylenediaminetetraacetate. This should be omitted when toxicity tests are conducted with metals. When tests are conducted with plankton or larvae, the EDTA should be omitted and the medium should be stripped of trace metals (Davey et al., 1970).

A freshwater dilution water is considered to be constant quality if the monthly ranges of the hardness, alkalinity, and specific conductance are less than 10% of their respective averages and if the monthly range of pH is less than 0.4 unit. A brackish or marine dilution water is considered to be constant quality if the weekly range of the salinity is less than 6 g/kg and if the monthly range of pH is less than 0.8 unit. Alternative freshwater dilution waters should be obtained from an uncontaminated well or spring if possible; only as a last resort should a dechlorinated water be used. If a dechlorinated water is used, at the beginning of STATIC tests and daily during FLOW-THROUGH tests either it must be shown that first instar daphnids can survive in it for 48 hours without food or residual chlorine must be measured. When possible, an alternative dilution water with a hardness of 44 mg/l as $CaCO_3$ should be used for BASIC tests with freshwater animals, with a salinity of 34 g/kg for BASIC tests with true marine stenohaline species, and with a salinity of 25 g/kg for BASIC tests with euryhaline species.

4. Effluent Tests

For EFFLUENT tests, the dilution water must be a representative sample of the receiving water obtained as close to the point of discharge as possible, but upstream of or outside the zone of influence of the effluent. The sample of the receiving water must not be aerated or altered in any way except that it may be filtered through a sieve or screen with 2 mm or larger holes. For STATIC EFFLUENT tests the dilution water should be obtained from the receiving water as close to the start of the test as possible, but never more than 96 hours prior to the beginning of the test. For FLOW-THROUGH EFFLUENT tests the dilution water may be obtained either by continuous sampling from the receiving water during the test or as one or more batches; if obtained in batches, the dilution water should not be obtained from the receiving water more than 96 hours prior

to the beginning of the test. If an acceptable dilution water cannot be obtained from the receiving water, aerated receiving water, or an uncontaminated, well-aerated surface, ground, or reconstituted water with hardness, alkalinity, and specific conductance within 25% and pH within 0.2 unit of those of the receiving water at the time of the test may be used. If a dechlorinated water is used, at the beginning of STATIC tests and daily during FLOW-THROUGH tests either it must be shown that first instar daphnids can survive in it for 48 hours without food or residual chlorine must be measured.

D. TEST ORGANISMS

1. Species

Whenever possible, BASIC tests should be conducted with the species listed in Table 3. If a recommended species is not available, organisms of the recommended genus should be used. The scientific name of the species used must be verified.

Whenever possible, EFFLUENT tests should be conducted with the most sensitive important species indigenous to or regularly stocked into the receiving water in the vicinity of the discharge. However, this species will depend on the receiving water, the composition of the effluent, etc., and is therefore generally difficult to identify without conducting tests with a variety of species. Therefore, tests are usually conducted with a readily available, commercially, or recreationally important species. Because centrarchids and salmonids are among the more sensitive species to many toxicants, are often available, are easy to handle, and are important in many receiving waters, they are often acceptable. The scientific name of the species used must be verified.

Table 3. RECOMMENDED SPECIES AND TEST TEMPERATURES

Recommended species[a]	Recommended test temperature (°C)[b]
Freshwater	
Vertebrates	
Coho salmon, *Oncorhynchus kisutch*	12
Rainbow trout, *Salmo gairdneri*	12
Brook trout, *Salvelinus fontinalis*	12
Goldfish, *Carassius auratus*	22
Fathead minnow, *Pimephales promelas*	22
Channel catfish, *Ictalurus punctatus*	22
Bluegill, *Lepomis macrochirus*	22
Invertebrates[b]	
Daphnids, *Daphnia magna* or *D. pulex*	17
Amphipods, *Gammarus lacustris*, *G. fasciatus*, or *G. pseudolimnaeus*	17 17
Crayfish, *Orconectes* sp., *Cambarus* sp., *Procambarus* sp., or *Pacifastacus leniusculus*	22 22
Stoneflies, *Pteronarcys* sp.	12
Mayflies, *Baetis* sp. or *Ephemerella* sp.	17
Mayflies, *Hexagenia limbata* or *H. bilinata*	22
Midges, *Chironomus* sp.	22
Marine and estuarine	
Vertebrates	
Sheepshead minnow, *Cyprinodon variegatus*	22
Mummichog, *Fundulus heteroclitus*	22
Longnose killifish, *Fundulus similis*	22
Silverside, *Menidia* sp.	22
Threespine stickleback, *Casterosteus aculeatus*	22
Pinfish, *Lagodon rhomboides*	22
Spot, *Leiostomus xanthurus*	22
Shiner perch, *Cymatogaster aggregata*	12
Pacific staghorn sculpin, *Leptocottus armatus*	12
Sanddab, *Citharichthys stigmaeus*	12
Flounder, *Paralichthys dentatus*, *P. lethostigma*	22
English sole, *Parophrys vetulus*	12

Table 3 (continued). RECOMMENDED SPECIES AND TEST TEMPERATURES

Recommended species[a]	Recommended test temperature (°C)[b]
Marine and estuarine Invertebrates[b]	
Shrimp, *Penaeus setiferus*, *P. duorarun*, or *P. aztecus*	22 22
Grass shrimp, *Palaemonetes* sp.	22
Shrimp, *Crangon* sp.	22
Oceanic shrimp, *Pandalus jordani*	12
Blue crab, *Callinectes sapidus*	22
Dungeness crab, *Cancer magister*	12

[a]The scientific name must be verified.

[b]Freshwater amphipods, daphnids, and midge larvae should be cultured and tested at the test temperature. Other invertebrates should be held and tested within 5° C of the temperature of the water from which they were obtained. They should be tested at the recommended test temperature if it is within this range; otherwise they should be tested at the temperature from the series 7, 12, 17, 22, and 27° C that is closest to the recommended test temperature and is within the allowed range.

2. Source

Usual sources of freshwater fish are private, state, and federal hatcheries. Whenever trout are to be used, certified disease-free fish (free of infectious pancreatic necrosis, furunculosis, kidney disease, and whirling disease) should be obtained if possible. Freshwater amphipods, daphnids, and midge larvae should be reared in the testing facility from laboratory cultures. Daphnids from cultures in which ephippia are being produced should not be used. The other suggested species are usually obtained directly from wild populations in relatively unpolluted areas. However, collecting permits may be required by local and state agencies. Organisms captured by electroshocking should not be used. All organisms in a test should be from the same source and as healthy and uniform in size and age as possible.

For BASIC tests analysis of organisms for pesticides, PCB's, phthalates, mercury, and the toxicant being tested is desirable. Organisms should not be used if the total concentration of organochlorine pesticides plus PCB's exceeds 0.3 µg/g (wet weight).

Although EFFLUENT tests should be conducted with a species that is indigenous to or stocked into the receiving water, the test organisms themselves do not have to be taken from the receiving water. Often it is difficult to obtain organisms in good condition from the receiving water and sometimes collecting permits are difficult to obtain. In addition, it is often difficult to determine whether or not motile organisms have been exposed to the effluent.

3. Size

a. Fish---Very young (not yet actively feeding), spawning or recently spent fish should not be used. The use of fish that weigh between 0.5

and 5.0 g each is usually desirable. Embryos and newly hatched fish are sometimes more sensitive than older stages and can be tested if appropriate precautions are taken. In any single test all fish should be from the same year class, and the standard length (tip of snout to end of caudal peduncle) of the longest fish should be no more than twice that of the shortest fish.

b. Invertebrates---Immature organisms should be used whenever possible. Among freshwater organisms, daphnids should be in the first instar; amphipods, stoneflies, and mayflies in an early instar; and midges in the second or third instar.

c. Amphibians---Young larvae should be used whenever possible.

4. Care and Handling

To avoid unnecessary stress, organisms should not be subjected to rapid changes in temperature or water quality. In general, aquatic organisms should not be subjected to more than a 3° C change in water temperature in any 12-hour period. Holding and acclimation tanks should be sterilized with an iodophor or with 200 mg of hypochlorite/liter for 1 hour, scrubbed well once during the hour, and then rinsed well between groups of test organisms. When organisms are first brought into the facility, they should be quarantined at least until they appear to be disease-free. To maintain organisms in good condition during holding and acclimation, crowding should be avoided and the dissolved oxygen concentration must be maintained between 60% and 100% saturation; gentle aeration may be used if necessary. Organisms should be fed at least once a day and tanks scrubbed at least twice a week. Organisms should be observed carefully during holding and acclimation for signs of disease, stress, physical damage, and mortality. Dead and abnormal individuals must be discarded.

Organisms should be handled as little as possible. When handling is necessary, it should be done as gently, carefully, and quickly as possible, so that the organisms are not unnecessarily stressed. Organisms that touch dry surfaces or are dropped or injured during handling must be discarded. Small dipnets are best for handling larger organisms. Such nets are commercially available, or can be made from small-mesh nylon netting, nylon or silk bolting cloth, plankton netting, or similar material. Nets coated with urethane resin are best for handling catfish. Smooth glass tubes with rubber bulbs should be used for transferring smaller organisms such as daphnids and midge larvae. Equipment used to handle aquatic organisms should be sterilized between uses with an iodophor, 200 mg of hypochlorite/liter, or 30% formalin plus 1% benzalkonium chloride. Hands should be washed or sterilized before handling or feeding test organisms.

5. Disease Treatment

Freshwater fish may be chemically treated to cure or prevent diseases by using the treatments recommended in Table 4, but if they are severely diseased, it is often better to destroy the entire lot. Until acceptable treatments have been proven effective, all other diseased animals should be discarded. Generally organisms should not be treated during the first 16 hours after they arrive at the facility because they are probably stressed due to collection or transportation and some are treated during transit. However, immediate treatment is necessary in some situations, such as treatment of bluegills for columnaris during hot weather. Tests must not be begun with treated organisms for at least 10 days after treatment for BASIC tests and at least 4 days after treatment for EFFLUENT tests. Tanks and test chambers which may be contaminated with undesirable microorganisms should be sterilized for 1 hour with an iodophor or with 200 mg of hypochlorite/liter.

Table 4. RECOMMENDED PROPHYLACTIC AND THERAPEUTIC TREATMENTS FOR FRESHWATER FISH[a]

Disease	Chemical	Concentration (mg/l)	Application
External bacteria	Benzalkonium chloride (Hyamine 1622[R])	1-2 AI[b]	30-60 min[c]
	Nitrofurazone (water mix)	3-5 AI	30-60 min[c]
	Neomycin sulfate	25	30-60 min[c]
	Oxytetracycline hydrochloride (water soluble)	25 AI	30-60 min[c]
Monogenetic trematodes, fungi, and external protozoa[d]	Formalin plus zinc-free malachite green oxalate	25 0.1	1-2 hours[c]
	Formalin	150-250	30-60 min[c]
	Potassium permanganate	2-6	30-60 min[c]
	Sodium chloride	15000-30000 2000-4000	5-10 min dip e,c
	Dexon[R] (35% AI)	20	30-60 min[c]
Parasitic copepods	Trichlorfon (Masoten[R])	0.25 AI	f

[a]These recommendations do not imply that these treatments have been cleared or registered for these uses. Appropriate state and federal regulatory agencies should be consulted to determine if the treatment in question can be used and under what conditions the uses are permitted. These treatments should be used only on fish intended for research. They have been found dependable, but efficacy against diseases and toxicity to fish may be altered by temperature or water quality. Researchers are cautioned to test treatments on small lots of fish before making large-scale applications. Prevention of disease is preferred, and newly acquired fish should be treated with the formalin-malachite green combination on three alternate days if possible. However,

Table 4 (continued). RECOMMENDED PROPHYLACTIC AND THERAPEUTIC TREATMENTS FOR FRESHWATER FISH

generally fish should not be treated on the first day they are in the facility. This table is merely an attempt to indicate the order of preference of treatments that have been reported to be effective. Before a treatment is used, additional information should be obtained from sources such as Davis (1953), Hoffman and Meyer (1974), Reichenbach-Klinke and Elkan (1965), Snieszko (1970), and van Duijn (1973).

[b]AI - active ingredient.

[c]Treatment may be accomplished by (1) transferring the fish to a static treatment tank and back to a holding tank; (2) temporarily stopping the flow in a flow-through system, treating the fish in a static manner, and then resuming the flow to flush out the chemical; or (3) continuously adding a stock solution of the chemical to a flow-through system by means of a metered flow or the technique of Brungs and Mount (1967).

[d]One treatment is usually sufficient except for "Ich," which must be treated daily or every other day until no sign of the protozoan remains. This may take 4-5 weeks at 5-10° C and 11-13 days at 15-21° C. A temperature of 32° C is lethal to Ich in one week.

[e]Minimum of 24 hours but may be continued indefinitely.

[f]Continuous treatment should be employed in static or flow-through systems until no copepods remain, except that treatment should not be continued for over 4 weeks and should not be used above 27° C.

6. Holding

After collection or transportation, invertebrates and amphibians must have been in holding or acclimation tanks for at least 10 days and fish for at least 14 days before they are used for BASIC tests; all test organisms must have been in holding or acclimation tanks for at least 2 days before they are used for EFFLUENT tests. They should be held under stable conditions of temperature and water quality in uncontaminated, constant-quality water in a flow-through system with a flow rate of at least two water volumes per day or, for EFFLUENT tests only, in a recirculating system in which the water flows through a carbon filter and an ultraviolet sterilizer. Water from a well or spring should be used for freshwater organisms whenever possible. Only as a last resort should a dechlorinated water be used. For BASIC tests water that may be contaminated with undesirable microorganisms should be passed through an ultraviolet sterilizer, and the un-ionized ammonia concentration in the holding tanks should be less than 20 µg/l. When possible, the organisms should be held in dilution water and at the temperature at which they are to be tested. During long holding periods, however, it is generally easier and safer to hold fish at lower temperatures rather than at higher temperatures because the metabolic rate and the number and severity of disease outbreaks are reduced. However, the recommended test temperatures listed in Table 3 are generally good temperatures at which to hold the respective organisms. Aquatic invertebrates should be held within 5° C of the temperature of the water from which they were obtained.

7. Acclimation

Freshwater amphipods, daphnids, and midge larvae should be acclimated to water quality and temperature by rearing them in the dilution water at the test temperature.

Other organisms can be acclimated (in a flow-through system with a flow rate of at least two water volumes per day for FLOW-THROUGH tests) simultaneously to the dilution water and test temperature after transferring an appropriate number of similar-length individuals from a holding tank to an acclimation tank. They should be acclimated to the dilution water by gradually changing the water in the acclimation tank from 100% holding water to 100% dilution water over a period of 2 or more days for BASIC tests and 24 or more hours for EFFLUENT tests. All organisms must remain in 100% dilution water for at least 2 days for BASIC tests and for at least 24 hours for EFFLUENT tests before they are used for tests. For BASIC tests water that may be contaminated with undesirable microorganisms should be passed through an ultraviolet sterilizer, and the un-ionized ammonia concentration in the acclimation tanks should be less than 20 µg/l. They should be acclimated to the test temperature by changing the water temperature at a rate not to exceed 3° C within 72 hours for BASIC tests and not to exceed 3° C within 24 hours for EFFLUENT tests until the allowable test temperature range is reached. They must be maintained for at least 2 days for BASIC tests and 24 hours for EFFLUENT tests at the allowable test temperature range before tests are begun with them. Longer acclimation times are generally desirable.

A group of organisms must not be used for a test if the individuals appear to be diseased or otherwise stressed or if more than 3% for BASIC tests or 5% for EFFLUENT tests die during the 48 hours immediately prior to the beginning of the test. If a group fails to meet these criteria, all individuals must be either discarded or treated, held an additional 10 days for BASIC test or 4 days for EFFLUENT tests, and reacclimated if necessary.

Young amphibian larvae and fish that have been actively feeding for less than about 20 days, amphipods, daphnids, and midge larvae must be fed, and all other insects may be fed, up to the beginning of the test. For

BASIC tests all other amphibian larvae and fish over 0.5 g each must not be fed for 96 hours, and all other invertebrates over 0.5 g each must not be fed for 48 hours, before the beginning of the test. For EFFLUENT tests all other amphibian larvae, fish, and invertebrates over 0.5 g each must not be fed for 48 hours before the beginning of the test.

E. TEST PROCEDURE

1. Experimental Design

For BASIC and EFFLUENT STATIC tests at least 10 organisms, and for EFFLUENT FLOW-THROUGH tests at least 20 organisms, must be exposed to each treatment, but they may be divided between two or more test chambers. The use of more organisms and replicate test chambers for each treatment is desirable. For BASIC FLOW-THROUGH tests at least 30 organisms must be exposed to each treatment, except that if replicate test chambers are used separately in the statistical analysis, at least 20 organisms must be exposed to each treatment. If replicates are used, they must be true replicates with no water connections between the replicate test chambers. Randomization of the treatments is desirable; if replicates are used, random assignment of one test chamber for each treatment in a row, followed by random assignment of a second test chamber for each treatment in another or an extension of the same row, is recommended rather than total randomization. A representative sample of the test organisms should be impartially distributed to the test chambers, either by adding one (if there are to be less than 11 organisms per container) or two (if there are to be more than 11 organisms per container) test organisms to each chamber, and then adding one or two more, and repeating the process until each test chamber has the desired number of test organisms in it. Alternatively, the organisms can be assigned either by random assignment of one organism to each test chamber, random assignment of a second organism to each test chamber, etc., or by total

randomization. It is often convenient to assign organisms to other containers and then add them to the test chambers all at once.

Every test requires a control which consists of the same dilution water, conditions, procedures, and organisms as are used in the remainder of the test. If any additive is present in any of the test chambers, an additive control is also required. This additive control is treated the same as the regular control except that the highest amount of additive present in any other test chamber is added to this test chamber. If the toxicant is a mixture or formulation, none of the ingredients of the mixture or formulation is considered an additive. A test is not acceptable if more than 10% (5% for BASIC FLOW-THROUGH tests) of the organisms die in any control in a test determining an LC50 or show the effect in a test determining an EC50.

It is desirable to repeat the test at a later time to obtain information on the reproducibility of the results of the test.

2. Dissolved Oxygen Concentration

Test solutions must not be aerated in the test chambers or in the toxicant delivery system. For BASIC STATIC tests the dissolved oxygen concentration in each test chamber must be between 60% and 100% saturation during the first 48 hours of the test and must be between 40% and 100% saturation after 48 hours. For BASIC FLOW-THROUGH tests the dissolved oxygen concentration in each test chamber must be between 60% and 100% saturation at all times during the test.

3. Test Temperature

For BASIC tests the test temperature must be selected from the series 7, 12, 17, 22, and 27° C. The actual test temperature must not deviate from the selected test temperature by more than 1° C at any time during

the test. The temperatures recommended in Table 3 should be used as the selected test temperatures for the species listed therein whenever possible. Other temperatures from the series can be used for studying the effect of temperature on the results of toxicity tests. For aquatic invertebrates the selected test temperature should be within 5° C of the temperature of the water from which they were obtained. They should be tested at the recommended test temperature if it is within this range; otherwise they should be tested at the temperature from the series 7, 12, 17, 22, and 27° C that is closest to the recommended test temperature and is within the allowed range.

For EFFLUENT STATIC tests the selected test temperature should be the temperature of the receiving water measured just outside the zone of influence of the effluent at noon (local time) on the day before acclimation begins, because the temperature at noon usually approximates the average temperature for the day. For aquatic invertebrates the selected test temperature should be within 5° C of the temperature of the water from which they were obtained, and as close to the measured noon temperature of the receiving water as possible. The actual test temperature must not deviate from the selected test temperature by more than 2° C at any time during the test.

The effect of temperature on the results of EFFLUENT STATIC tests can be determined by conducting additional toxicity tests with different test temperatures. When special tests are conducted to collect additional information about an effluent, it must be remembered that the characteristics of the effluent and the receiving water may vary significantly within short periods of time. Thus tests to determine the effect of temperature should all be conducted at the same time on the same samples of the effluent and the receiving water.

For EFFLUENT FLOW-THROUGH tests the actual test temperature must always be between the daily low and the daily high temperature of the receiving water measured at the time of the test just outside the zone of influence of the effluent. For aquatic invertebrates the test temperature should always be within 5° C of the temperature of the water from which they were obtained and should always be between the daily low and the daily high temperature of the receiving water measured at the time of the test just outside the zone of influence of the effluent if possible. The actual test temperature may be constant throughout the test or may fluctuate within the allowable test temperature range with the temperature of the receiving water.

4. Loading

The grams of organism per liter of solution in the test chambers must not be so high that it affects the results of the test. Therefore the loading must be limited to insure that the concentration of dissolved oxygen and toxicant is not decreased below acceptable levels, that the concentration of metabolic products does not increase above acceptable levels, and that the organisms are not stressed due to crowding. For STATIC tests with the species listed in Table 3 the loading in the test chambers must not exceed 0.8 g/liter at or below the temperatures specified as the recommended test temperatures and 0.4 g/liter at higher temperatures. For FLOW-THROUGH tests with the species listed in Table 3 the loading in the test chambers must not exceed 2 g per liter of test solution passing through the test chamber in 24 hours and must not exceed 20 g/liter of test solution in the test chamber at any time at or below the temperatures specified as the recommended test temperatures; at higher temperatures the loading must not exceed 1 g/(liter/day) or 10 g/liter. (The recommended test temperatures listed in Table 3 have no bearing on the temperatures at which EFFLUENT tests should be conducted.)

For BASIC STATIC tests lower loadings must be used if the concentration of dissolved oxygen does not remain above 60% saturation for the first 48 hours of the test and above 40% saturation after 48 hours.

For BASIC FLOW-THROUGH tests lower loadings must be used if necessary to meet the following three criteria at all times during the test in each test chamber:

a. the concentration of dissolved oxygen must not fall below 60% saturation;
b. the concentration of un-ionized ammonia must not exceed 20 µg/l; and
c. the concentration of toxicant must not be lowered by more than 20% because of uptake by the test organisms.

For EFFLUENT tests, when the concentration of dissolved oxygen in the dilution water is less than 60% saturation at the beginning of the test, lower loadings than those specified above should be used. If the dissolved oxygen concentration is less than 60% saturation in any test chamber at any time during an EFFLUENT test, it may also be desirable to conduct an additional toxicity test by a modified procedure by slowly bubbling air or oxygen through the solutions in the test chambers during the test. When tests are conducted by such modified methods, the exact methodology must be described in detail in all reports of the results of the test. In order to determine the effect of the test organisms on the dissolved oxygen concentration during EFFLUENT tests, the dissolved oxygen concentration should be measured in duplicate test chambers that do not contain test organisms.

Comparable loadings should be used for other species.

5. Toxicant

For BASIC tests the toxicant should be added to the dilution water (in the toxicant delivery system for FLOW-THROUGH tests) without the use of any solvents or other additives, except water, if possible. If additives other than water are necessary, the amount used must be kept to a minimum. Hydrochloric acid, nitric acid, potassium hydroxide, sodium hydroxide, and sulfuric acid may be used to prepare aqueous stock solutions, but they may affect the pH of the test solutions appreciably. Acetone, dimethylformamide (DMF), ethanol, methanol, and triethylene glycol may be used to prepare stock solutions, but the concentration of solvent in any test solution must not exceed 0.5 ml/liter in BASIC STATIC tests and 0.1 ml/liter in BASIC FLOW-THROUGH tests. The stability of the toxicant in the stock solution should be determined.

For EFFLUENT tests the toxicant is a sample of an effluent. The sample of the effluent must not be aerated or altered in any way except that it may be filtered through a sieve or screen with 2 mm or larger holes. Samples must be covered at all times and violent agitation must be avoided. Undissolved materials must be uniformly dispersed by gentle agitation immediately before any aliquot of the sample is taken for use, especially in the headbox for EFFLUENT FLOW-THROUGH tests. The timing of the test and the collection of samples should be based on an understanding of the short- and long-term operations and schedules of the discharger if possible.

For EFFLUENT STATIC tests separate tests generally should be conducted on at least two grab samples and more tests may often be desirable, especially if there are known sources of variability such as process changes. Tests on composite samples may be desirable in some cases. Tests should be begun as soon as possible, but must be begun within 8 hours, after the sample is obtained. The temperature of the sample should be adjusted to

the test temperature (± 2° C) and maintained at that temperature until portions are added to the dilution water. Often it is convenient to store the sample in the constant temperature water bath or area in which the test chambers are placed during the test.

For EFFLUENT FLOW-THROUGH tests on effluents that are discharged continuously for over 96 hours, the sample of the effluent must be taken continuously from the discharge line and introduced directly into a small effluent headbox that feeds the toxicant delivery system. If the discharge rate is not reasonably constant, flow-proportional continuous sampling may be desirable. For effluents that are only discharged in batches, a grab sample must be used and the test must begin within 8 hours after the sample is obtained. The temperature of the sample should be adjusted to be within the allowable test temperature range before it is added to the dilution water.

Special EFFLUENT tests may be conducted on altered or treated samples of the effluent or on other samples to obtain additional information concerning the toxicity of the effluent. For example, a special EFFLUENT STATIC test can be conducted by mixing effluent with dilution water and letting the solutions age for a period of time, such as 24 hours, before adding the test organisms to determine if toxicity increases or decreases with time. When special tests are conducted, the exact methodology must be described in all reports of the results of the tests.

6. Beginning the Test

STATIC tests are begun either by (a) adding toxicant to the test chambers 18 to 24 hours after the test organisms are added or (b) adding test organisms to the test chambers within 30 minutes after the toxicant is added to the dilution water. The first alternative (a) allows the test organisms to partially acclimate to the test chambers and precludes loss

of toxicant due to hydrolysis, sorption, or evaporation prior to exposure of the test organisms. The second alternative (b) conserves dissolved oxygen and prevents the exposure of test organisms to the toxicant before it is evenly dispersed; this alternative must be used when tests are conducted on aged solutions of a toxicant in dilution water and when tests are conducted with daphnids or midge larvae. A gentle swirl with a glass rod is usually sufficient to disperse the toxicant.

FLOW-THROUGH tests are begun either by (a) placing test organisms in the test chambers after the test solutions have been flowing through the test chambers long enough so that the toxicant concentrations are constant or (b) activating the toxicant metering device in the toxicant delivery system several days after the test organisms were placed in test chambers that had dilution water flowing through them. The first alternative (a) allows the investigator to study the behavior of the toxicant and the toxicant delivery system immediately prior to the beginning of the test, whereas the second alternative (b) allows the test organisms to partially acclimate to the test chambers before the beginning of the test.

7. Feeding

The test organisms must not be fed while in the test chambers.

8. Duration

A test begins when the test organisms are first exposed to the toxicant. Daphnids and midge larvae must be exposed to the toxicant for at least 48 hours. All other organisms must be exposed for 96 hours in BASIC STATIC tests, for 48 to 96 hours in EFFLUENT STATIC tests, and for at least 96 hours in all FLOW-THROUGH tests. When BASIC FLOW-THROUGH tests are conducted with larger organisms (over 0.5 g each), it is usually desirable to determine the shape of the toxicity curve, i.e., LC50 or EC50 vs. time, throughout an 8-day exposure.

9. Biological Data

The number of dead or affected organisms in each test chamber must be counted every 24 hours after the beginning of the test. More observations are desirable, especially near the beginning of the test.

For BASIC FLOW-THROUGH tests the number should be counted at enough appropriate times during the test to define the shape of the toxicity curve. A suggested schedule is to count the number of dead or affected organisms in each test chamber 3, 6, 12, and 24 hours after the beginning of the test and twice a day thereafter to the end of the test. It is more important to obtain data that define the shape of the toxicity curve than it is to obtain data at prespecified times other than every 24 hours.

Dead organisms must be removed as soon as they are observed in STATIC tests and at least once every 24 hours in FLOW-THROUGH tests.

Death is the adverse effect most often used to study acute toxicity with aquatic organisms. The criteria for death are usually the lack of movement, especially the absence of gill movement in fish, and the lack of reaction to gentle prodding. However, because death is not easily determined for some invertebrates, an EC50 is often determined rather than an LC50. The effect generally used for determining an EC50 with daphnids and midge larvae is immobilization, which is defined as the lack of movement except for minor activity of appendages. The effects generally used for determining an EC50 with crabs, crayfish, and shrimp are immobilization and loss of equilibrium. Other effects can be used for determining an EC50, but the effect and its definition must always be

reported. General observations on such things as erratic swimming, loss of reflex, discoloration, changes in behavior, excessive mucus production, hyperventilation, opaque eyes, curved spine, hemorrhaging, molting, and cannibalism should be reported.

The weights and standard lengths of the test organisms should be determined by measuring representative organisms before the test or the control organisms after the test. Organisms that are to be used in a test must not be weighed or measured after acclimation has begun.

10. Chemical and Physical Data

a. BASIC STATIC tests---

If a freshwater dilution water is used, its hardness, alkalinity, pH, and specific conductance must be measured. If a brackish or marine dilution water is used, its salinity and pH must be measured. Measurement of suspended solids and TOC or COD is desirable. The dissolved oxygen concentration must be measured at the beginning of the test and every 48 hours thereafter to the end of the test in the control and the high, medium, and low toxicant concentrations as long as test organisms are present. The pH should be measured at the beginning and end of the test in the control and the high, medium, and low toxicant concentrations. If possible, the concentration of toxicant should be measured at the beginning and end of the test in all test chambers. Measurement of degradation products of the toxicant is desirable.

b. BASIC FLOW-THROUGH tests---

(1) Analytical method for toxicant analysis---When the identity of the toxicant is known, the concentration of toxicant in the test chambers

must be measured. When the concentration is not measured, the usefulness of the flow-through technique may be greatly diminished. The analytical method for measuring the concentration of toxicant must be validated before the beginning of the test. At a minimum, a measure of the accuracy of the method must be obtained by using the method of known additions with dilution water from a tank containing test organisms; on each of two separate days three samples must be analyzed at the next to the lowest toxicant concentration that will be used in the toxicity test. It is also desirable to study the accuracy and precision of the analytical method by use of reference or split samples or interlaboratory studies and by comparison with alternative, preferably reference or corroborative, methods of analysis. The accuracy of standard solutions should be checked against other standard solutions whenever possible.

An analytical method is not acceptable if likely degradation products of the toxicant, such as hydrolysis and oxidation products, give positive or negative interferences, unless it is shown that such degradation products are not present in the test chambers during the test. In general, atomic absorption spectrophotometric methods for metals and gas chromatographic methods for organic compounds are preferable to colorimetric methods.

In addition to measuring the total concentration of toxicant in the test chambers, it is usually desirable to make measurements of either the "dissolved" or the "undissolved" fraction of the toxicant. Especially for inorganic substances the "dissolved" fraction is usually defined and determined as that which passes through a 0.45 micron membrane filter. Glass filter holders are best for organic toxicants, but plastic holders are best for metals. Filters and their holders must always be prewashed by filtering distilled water and then filtering a portion of the solution of interest before the final filtration is performed. The final portion of the filtered distilled water should be analyzed for toxicant to make sure

that the filter is not contaminated with toxicant. The sample must be filtered within 30 minutes after it is taken from the test chamber.

Whenever samples from a toxicity test are analyzed, at least one reagent blank must also be analyzed, if appropriate. Also, at least one sample for the method of known additions must be prepared by adding toxicant to water from a control test chamber to match the next to the lowest toxicant concentration used in the toxicity test.

(2) Behavior of the toxicant---Data should be available or should be generated to show that under the conditions of the test at the flow rate used (a) no more than 20% of the toxicant at the next to the lowest concentration will degrade in or volatilize from the test chambers or both; and (b) at the loading used, no less than 80% of the toxicant that would be present in the next to the lowest concentration without test organisms in the test chamber will be present with test organisms in the test chamber. The latter problem can be alleviated by reducing the loading, and both problems by using a faster flow rate.

(3) Toxicant measurements during the test---It is desirable to measure the concentration of toxicant in the test chambers as often as practical during the test. At a minimum the concentration of toxicant must be measured in (a) each test chamber at least once during the test; (b) at least one test chamber at the next to the lowest toxicant concentration at least once every 24 hours during the test; and (c) at least one appropriate test chamber whenever a malfunction is detected in any part of the toxicant delivery system. For replicate test chambers at the same toxicant concentration, the highest measured concentration divided by the lowest measured concentration must be less than 1.2. If it is not, the toxicant delivery system should be checked, and additional samples from the proper test chambers should be analyzed to determine if the

sampling or analytical methods are not precise enough. In addition, the measured concentration of toxicant in any test chamber must be no more than 30% higher or lower than the concentration calculated from the composition of the stock solution and the calibration of the toxicant delivery system. If the difference is more than 30%, measuring the concentration of toxicant in the test solution flowing into the test chamber will indicate whether the problem is in the toxicant delivery system or in the test chamber. Measurement of degradation products of the toxicant is desirable.

(4) Analyses of water quality---Certain measurements must be performed at least once every 30 days or at the beginning of the test, if data are available to show that the quality of the dilution water is constant, and daily if such data are not available. For a freshwater dilution water hardness, alkalinity, pH, specific conductance, TOC or COD, and suspended solids must be measured, and for a brackish or marine dilution water salinity, pH, TOC or COD, and suspended solids must be measured. For fresh waters it is also desirable to measure the concentration of calcium, magnesium, sodium, potassium, chloride, and sulfate. The dissolved oxygen concentration must be measured at the beginning of the test and every 48 hours thereafter to the end of the test in the control and the high, medium, and low toxicant concentrations as long as test organisms are present. The pH should be measured at least once in the control and the high, medium, and low toxicant concentration.

c. EFFLUENT STATIC tests---

The dissolved oxygen concentration must be measured at the beginning of the test and every 48 hours thereafter to the end of the test in the control and the high, medium, and low effluent concentrations as long as test organisms are present. The pH and specific conductance must be

measured at the beginning of the test in the control and the high, medium, and low effluent concentrations.

d. EFFLUENT FLOW-THROUGH tests---

The dissolved oxygen concentration, pH, and specific conductance must be measured at the beginning of the test and every 24 hours thereafter to the end of the test in the control and the high, medium, and low effluent concentrations as long as test organisms are present.

e. All Tests---

Temperature must be recorded at least hourly throughout acclimation and throughout the test in at least one test chamber. Additional measurements on dilution water, test solutions, and effluent samples are often desirable. When water samples are taken from test chambers, they must be taken midway between the top, bottom, and sides of the test chambers and should not include any surface scum or material stirred up from the bottom or sides.

Methods used for analysis of water quality must be those specified in the latest edition of the Annual Book of Standards, Part 31 (American Society for Testing and Materials, 1974) or Methods for Chemical Analysis of Water and Wastes (U. S. Environmental Protection Agency, 1974). Residual chlorine can be measured to 3 µg/l using a modified amperometric method (Andrew and Glass, 1974). The concentration of un-ionized ammonia can be calculated from the concentration of total ammonia, pH, and temperature according to Table 5. Salinity should be measured with a conductivity salinometer or by a chlorinity titration.

11. Range-finding Test

Unless the approximate toxicity of the toxicant is already known, it is usually desirable to conduct a range-finding test to determine the toxicant

Table 5. PERCENTAGE OF AMMONIA THAT IS UN-IONIZED IN DISTILLED WATER AT DIFFERENT TEMPERATURES AND pH'S[a]

Temperature (°C)	pH								
	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5	10.0
7	0.01	0.05	0.15	0.46	1.45	4.44	12.8	31.7	59.5
12	0.02	0.07	0.22	0.68	2.13	6.44	17.9	40.8	68.5
17	0.03	0.10	0.32	1.00	3.08	9.14	24.1	50.2	76.1
22	0.04	0.14	0.45	1.43	4.39	12.7	31.5	59.2	82.1
27	0.06	0.21	0.65	2.03	6.15	17.2	39.6	67.4	86.8

[a]From Thurston, Russo, and Emerson (1974).

concentrations that should be used in the definitive test. Generally groups of five organisms are exposed to three to five widely spaced toxicant concentrations and a control for 24 to 96 hours using the static or flow-through techniques. The greater the similarity between the range-finding test and the definitive test, the more useful the results of the range-finding test will be.

Meaningful range-finding tests may often be difficult to conduct for EFFLUENT tests because the characteristics of the effluent and the receiving water may vary significantly within short periods of time. However, many nonchlorinated effluents have an LC50 between 2% and 100%. If a range-finding test is to be conducted with the same grab sample of the effluent with which a definitive EFFLUENT test is to be conducted, the range-finding test can last 8 hours at the most.

12. Definitive Test

For the determination of an LC50 or an EC50, a control and at least five concentrations of toxicant in a geometric series should be used. More treatments are desirable to insure the acceptability of the test and to provide additional data for various lengths of exposure. A definitive test must meet both of the following criteria so that the LC50 or EC50 can be calculated with reasonable accuracy:

a. Except for the controls, the concentration of toxicant in each treatment must be at least 60% of the next higher one for BASIC tests and at least 50% of the next higher one for EFFLUENT tests.

b. One treatment other than the control must have killed or affected less than 35% of the organisms exposed to it, and one treatment must have killed or affected more than 65% of the organisms. This requirement does not apply to EFFLUENT tests if 100% effluent does not kill or affect more than 65% of the organisms exposed to it.

If an LC or EC near the extremes of toxicity is to be calculated, such as an LC10 or an EC90, at least one treatment must have killed or affected a percentage of test organisms, other than 0% and 100%, near the percentage for which the LC or EC is to be calculated. This requirement might be met in a test to determine an LC50 or an EC50, but special tests with appropriate toxicant concentrations will often be necessary.

Other ways of providing information concerning the extremes of toxicity are to report the highest concentration of toxicant that actually killed or affected no greater a percentage of the test organisms than did the control treatment in the toxicity test or to report the lowest concentration of toxicant that actually killed or affected all of the test organisms exposed to it. These alternatives are normally more informative than reporting a result such as an LC2 or an EC98 unless several partial kills or effects are obtained close to 2% or 98%.

13. Calculations

For each set of data the LC50 or EC50 and its 95% confidence limits must be calculated on the basis of (a) the measured initial concentrations of toxicant, if available, or the calculated initial concentrations for BASIC STATIC tests, (b) the average measured concentrations of toxicant, if available, or the calculated average concentrations for BASIC FLOW-THROUGH tests, (c) the calculated initial volume per cent of the effluent in the test solutions for EFFLUENT STATIC tests, and (d) the calculated average volume per cent of the effluent in the test solutions for EFFLUENT FLOW-THROUGH tests. The "volume per cent" equals "(100 X volume of effluent)/(volume of effluent plus volume of dilution water)." If other LC or EC values are calculated, their 95% confidence limits must also be calculated. A variety of methods can be used to calculate an LC or EC (Finney, 1964, 1971), but the most widely used are the probit, logit, moving average, and Litchfield-Wilcoxon (1949) methods. The percentage

of the test organisms that die or show the effect in the control treatment must not be used in the calculation of the results.

To be sure that the calculations are being performed correctly, results calculated for the hypothetical sets of data in Table 6, assuming ten test organisms per treatment, should fall within the ranges of acceptable values given therein.

F. REPORTS

A report of the results of a test must include the following:

1. name of method, investigator, and laboratory, and date test was conducted;
2. for BASIC tests a detailed description of the toxicant, including its source, composition, known physical and chemical properties, and any additives used, and for EFFLUENT tests a detailed description of the effluent, including its source, date, and time of collection, composition, known physical and chemical properties, and variability;
3. the source of the dilution water, its chemical characteristics, and a description of any pretreatment, and for EFFLUENT tests the date and time of collection;
4. detailed information about the test organisms, including scientific name, standard length, weight, age, life stage, source, history, observed diseases, treatments, and acclimation procedure used;
5. a description of the experimental design and the test chambers, the depth and volume of solution in the test chambers, the way the test was begun, the number of organisms per treatment, the loading, the lighting, and for FLOW-THROUGH tests a description of the toxicant delivery system and the flow rate as the average number of water volumes of test solution passing through each test chamber in 24 hours;
6. definition of the criterion used to determine the effect and a summary of general observations on other effects or symptoms;
7. percentage of organisms that died or showed the effect in the control treatment;

Table 6. HYPOTHETICAL SETS OF DATA AND CALCULATED RESULTS

Table 6a. Percentage mortality

Data set	Concentrations (µg/l)						
	100	60	36	22	13	7.8	Control
A	100	100	100	10	0	0	0
B	100	100	100	70	0	0	0
C	100	100	40	10	0	0	0
D	100	100	70	20	0	0	0
E	100	100	30	20	0	0	0

Table 6b. Range of acceptable values

Data set	LC50	95% Confidence limits	
		Lower	Upper
A	25.5-27.5	21.1-24.0	28.6-30.8
B	19.0-21.5	14.7-19.4	22.9-24.5
C	35.5-37.2	26.1-30.7	43.4-45.3
D	29.4-30.0	23.5-23.9	36.3-37.4
E	35.4-40.5	28.1-30.8	44.5-46.0

8. for daphnids and midge larvae, the 24- and 48-hour, and for all other organisms, the 24-, 48-, and 96-hour LC50 or EC50 values and their 95% confidence limits and the method used to calculate them (if 100% effluent does not kill or affect more than 65% of the test organisms, report the percentage of test organisms killed or affected by various concentrations of the effluent), and for BASIC FLOW-THROUGH tests enough other LC50 or EC50 values to define the shape of the toxicity curve;
9. methods used for, and the results of, all chemical analyses of water quality and toxicant concentration, including validation studies and reagent blanks;
10. the average and range of the acclimation temperature and the test temperature;
11. anything unusual about the test;
12. any deviation from these methods; and
13. any other relevant information.

SECTION V

DISCUSSION

These methods concern acute toxicity tests with fish, macroinvertebrates, and amphibians, but many of the considerations dealt with apply directly to other kinds of toxicity tests and aquatic organisms. Whenever toxicity tests are conducted with aquatic organisms, the methods presented here should be followed as closely as possible. Of necessity, some of the recommendations are based on a professional consensus rather than on scientific data. Therefore, the committee encourages additional research on methodology and hopes that the present methods will serve as the starting point for such work.

Many reasons exist for conducting toxicity tests with aquatic organisms and various organisms, endpoints, and techniques are commonly used. Since no detailed method can satisfy all needs, four different methods have been written, identified by titles that clearly specify a definite set of procedures. Use of these methods for special purposes may require modification or specification of additional details, such as choosing one particular species.

Since not all details are covered in these methods, the successful execution of these tests will require some training or experience in aquatic toxicology or aquatic biology or both as well as a familiarity with the material in many of the references, especially American Public Health Association (1971) and Sprague (1969, 1973). It is essential to

conduct tests so that they meet specific needs, but the present methods should cover most situations. To obtain the most meaningful data, the investigator should consult with an aquatic biologist, analytical chemist, biometrician, and aquatic toxicologist before the test.

The static technique provides the easiest measure of toxicity and is often the only practical means of estimating the influence of variables such as temperature and water quality on the results of toxicity tests. The static technique should not be used for exposures lasting longer than 96 hours. Flow-through tests can last longer than 96 hours because this technique provides for continual addition of test solution to the test chambers, maintenance of the dissolved oxygen and toxicant concentrations and pH at desired levels, and removal of degradation and metabolic products. The flow-through technique should be used when other than the static is desired, although the use of the recirculation and renewal techniques may be justified in some cases.

Probably the most important consideration in the preparation for toxicity tests with aquatic organisms is obtaining an adequate supply of water of acceptable quality. Reconstituted water can be prepared from almost any water, but the cost of preparing it from low quality water can be very high. Continuous treatment of water for flow-through tests can be very costly. For tests with freshwater organisms municipal water supplies often contain unacceptable concentrations of copper, lead, zinc, fluoride, and chlorine or chloramines. Metals can be removed with chelating resins. Sodium bisulfite should be better for dechlorinating water than sodium sulfite; both are much more reliable than carbon filters, especially for removing chloramines. For acute tests with freshwater animals it is always desirable to continuously biomonitor the quality of the dilution water by determining daily if first instar daphnids can survive in it for 48 hours without food. The results of tests conducted in freshwater

dilution water prepared from chlorinated water must almost always be suspect unless the acceptability of the water has been proven with first instar daphnids. Systems have been developed to provide acceptable sea water from surface water sources (Clark and Clark, 1964; Wood, 1965; Tenore and Huguenin, 1973; White et al., 1973).

The recommended reconstituted waters have been shown to be acceptable to aquatic organisms. Daphnids can survive and reproduce satisfactorily in the soft and the hard reconstituted fresh waters (H. Sanders, Fish-Pesticide Research Laboratory, Columbia, Missouri, personal communication; R. Winner, Miami University, Oxford, Ohio, personal communication). The recommended sea water is acceptable for artemia (Zillioux et al., 1973) and oyster larvae (Zaroogian et al., 1969; Calabrese et al., 1973).

Although some general information on the care and handling of aquatic animals is available (Brauhn and Schoettger, 1974; National Academy of Sciences, 1973a, 1973b, 1974a, 1974b), a continuing problem in aquatic toxicology is the lack of detailed information concerning quality control of test organisms. This deficiency stems from a lack of information on the effects of nutrition, diseases, acclimation, and other stresses on aquatic organisms. Most investigators would rather not use organisms that have been treated to prevent or cure diseases; they usually find, however, that it is impractical to try to use only untreated organisms, partly because most organisms are treated during transportation. In spite of probable limitations, some investigators favor the use of a reference toxicant as a measure of the quality of test organisms (Marking, 1966). Not enough information is available to recommend any one compound, although antimycin has been found useful for detecting stressed freshwater fish (Hunn, Schoettger, and Whealdon, 1968; L. Marking, Fish Control Laboratory, La Crosse, Wisconsin, personal communication). Complete

acclimation may take considerably longer than even that specified for basic tests; therefore, acclimation times longer than the minimum specified should be used when possible.

The recommended species were selected on the basis of availability, importance, past use, and ease of handling in the laboratory, and were chosen primarily to encourage uniformity so that much information becomes available about a few species rather than a little information about many species. Much work needs to be done to identify species that are important in aquatic environments over wide geographical regions, are readily available, and can be used easily in toxicity tests. Some organisms, especially oyster embryos, are not listed because their use in toxicity tests requires special methodology (Woelke, 1972; Calabrese et al., 1973).

Test organisms should not be fed during acute toxicity tests and should not be fed for a time before tests when possible because fecal matter and uneaten food can affect the biological activity of some toxic agents and increase biological oxygen demand. These problems are most severe with the static technique, but can be important even with the flow-through technique. Because daphnids and midge larvae usually cannot live much longer than 48 hours without food, these organisms should be fed up to the beginning of the test. Withholding food for up to 12 days from healthy fish and macroinvertebrates weighing over 0.5 g each should not affect the results of toxicity tests.

Among the many factors taken into account in the selection of the recommended test temperatures were the acceptable temperatures for holding individual species in the laboratory; the solubility of oxygen in water; the rates of metabolism, degradation, and volatilization; the consumption of dissolved oxygen; the effect of temperature on the results of toxicity tests; the prevelance of diseases; past practice; and the relationship of

test temperature to room temperature. The series 7, 12, 17, 22, and 27° C was chosen because it better suits more organisms than does any other series. Although the recommended test temperatures were deemed to be very acceptable to the organisms, they should also be convenient for use by most investigators. Optimum temperatures for growth were not a major consideration in the selection of recommended test temperatures because organisms in nature spend very little of their time at the optimum temperature, it would be necessary to know the optimum temperature for each life stage of each organism used in tests, organisms can function well over a range of temperatures, diseases are often much more prevalant at optimum temperatures than at lower acceptable temperatures, and the use of optimum temperatures as test temperatures would be very inconvenient for many investigators. An additional consideration for freshwater fish is that they function well at temperatures 5° C higher and lower than the recommended test temperatures so the effect of temperature on the results of toxicity tests can be studied at 7, 12, and 17° C for cold water freshwater fish and at 17, 22, and 27° C for warm water freshwater fish. Other temperatures have been recommended for other kinds of tests with some of these organisms (National Academy of Sciences, 1973a).

The methods are applicable to freshwater, estuarine, and marine animals. The large amount of information available on freshwater organisms and the small amount on estuarine and marine organisms are a direct reflection of the amount of research that has been done with the various kinds of aquatic life. It is hoped that gaps in available information will be filled soon.

The biological testing of aqueous effluents has some advantages and some disadvantages when compared with chemical testing. Therefore, the various methods should be used to complement one another. In biological tests aquatic organisms integrate the synergistic and antagonistic effects of all components over the duration of the exposure. Even though one biological

test normally costs more than one chemical test, reasonable coverage with biological tests may not cost more than reasonably complete coverage with chemical tests when all levels of all potentially harmful toxic agents are taken into account. All tests have their limitations and no amount of biological and chemical testing can provide enough data to guarantee complete protection of the receiving water as a natural resource. However, a reasonable amount of testing can provide a basis for a plausible amount of protection.

Usually a flow-through test involving continuous sampling of both the effluent and the receiving water is considered the best way to conduct toxicity tests on aqueous effluents because in this way the test organisms are exposed to all occurring levels of all toxic agents. The flow-through technique is particularly advantageous over the static technique for effluents that are variable, have a high biological oxygen demand, or contain degradable or volatile toxicants. All three of these problems tend to be less important with treated effluents than with untreated ones. However, the static technique is often used for effluent testing because it generally provides useful data at a lower cost than does the flow-through technique.

Some effluent testing must be tailored to meet the specific requirements of regulatory agencies. In such cases the requirements of the regulatory agency should be followed regardless of any other recommendations or an agreement should be reached before the tests are started.

An attempt was made to balance scientific considerations against practical considerations and the reliability of the results. The major consideration was that the common uses of the results do not require or justify stricter requirements than those set forth herein. The requirements for acceptable static tests are lenient because of the inherent limitations of the

technique. Special considerations for effluent tests include the variability of effluents and receiving waters, the fact that the tests will often be conducted in field situations and in mobile laboratories, and the uses to which the results will be put. Although any of the tests can be improved by using more test organisms, etc., the committee feels that the requirements as presented will meet the needs of most situations. The methods are designed to insure that the results will be accurate and precise enough for the majority of situations requiring acute toxicity tests. A decision to conduct more precise tests will usually be based on personal preference rather than on a practical need for more precise results. The 95% confidence limits must be reported because they provide a good indication of the precision of a test. The ranges of acceptable values given in Table 6 were derived by comparing the values calculated by various people using a variety of methods of calculation. Ranges are given because there are no single correct values. When valid methods of calculation are correctly used, values within the ranges given should be obtained.

SECTION VI

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